

THE INITIATING AND PROMOTING ELEMENTS IN TUMOR PRODUCTION

AN ANALYSIS OF THE EFFECTS OF TAR, BENZPYRENE, AND METHYLCHOLANTHRENE ON RABBIT SKIN

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PLATES 7 TO 9

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The growths which tarring first calls forth on rabbit skin are nearly all benign and, though often becoming huge, are possessed of so little innate vigor as to be wholly dependent for success upon favoring local conditions. Nevertheless they are genuine neoplasms as experimental inquiry has shown (1). Tarring provides them with the conditions needed for growth, but after it is discontinued the tumors all more or less gradually disappear unless some other aid is forthcoming (1) or they become cancerous, as only occasionally happens. Plainly the carcinogen acts in two ways: it changes normal cells into neoplastic cells and encourages the multiplication of these latter. Its ability to effect neoplastic change exceeds its promoting power however, and as result the skin soon comes to contain several times as many tumor cells as manifest themselves by producing growths (2). Many of the hidden entities, which ordinarily would never come to anything as the late findings show, can be induced to assert themselves and form visible tumors by agents or conditions which do not themselves initiate neoplastic change but which stimulate normal epidermal cells to proliferate,—turpentine, irritant exudates, wound healing, for example. These facts lead one to ask whether the action of other carcinogens may not be also of a twofold sort, initiating and promoting. If this is the case their relative effectiveness must depend to a more or less considerable extent on how far they or the conditions they induce act to encourage, or perhaps frustrate, the cells they have rendered neoplastic. There is the more need to inquire into this matter because the activity of carcinogenic agents is measured nowadays in terms of the lump result, literally, with no question raised as to how this comes about. The present paper reports an experimental analysis of the factors entering into the effectiveness of tar, benzpyrene, and methylcholanthrene when applied to rabbit skin.

Benzpyrene is the carcinogen to which the effectiveness of coal tar has been attributed. It is present in only minute amount (3) and yet tar gives rise to cutaneous rabbit tumors much sooner than does benzpyrene when applied in quantity. The paradox has excited wide remark and has led some workers to

suppose that additional carcinogens must be present in tar. However, this may be¹ there is reason to ask whether the effectiveness of tar is not largely due to a superior ability to promote tumor formation. Methylcholanthrene seemed a good choice for study because it is one of the most powerful of tumor-producing substances.

A considerable literature exists on the agents and influences which aid or hinder the experimental production of tumors, but the question has seldom been raised of whether the carcinogenic agents act in other ways besides bringing on neoplastic changes. In 1926, Sobolewa (5) reported that the appearance of tar tumors is hastened by applications of mustard oil, a material non-carcinogenic in itself. Twort and Twort (6) and Shear (7) have since made observations of similar import with other substances, and Berenblum (8) has discovered inhibitory as well as furthering agents and has pointed out that the influence of the latter should be discriminated from actual carcinogenesis. Haddow (9) has concluded that benzpyrene and methylcholanthrene depress the activity of cells with result that some undergo mutant alterations which find expression in the neoplastic state; and he and his associates have shown that the hydrocarbons mentioned, as also many other carcinogenic substances, may retard the growth of existing tumors of various kinds (10). A host of workers have investigated the influence of solvents upon the effectiveness of carcinogenic substances, and the fact has come to be recognized that often they largely determine the tumor yield. It is generally assumed that this is because some of them bring the carcinogens into more direct and prolonged contact with the tissues than others do.

General Plan

In order to learn whether benzpyrene and methylcholanthrene have any effect to speed or retard the formation of tumors by the cells they have rendered neoplastic it is essential to test them upon responsive elements. The benign growths which tar calls forth on rabbit skin are highly sensitive to its promoting influence, and the supposition seemed warranted that benzpyrene and methylcholanthrene would give rise to tumors equally revealing. This has proved to be the case.

For tests of the power of the carcinogens to initiate neoplastic change it was necessary to have a means whereby tumor cells could be induced to form visible growths. The stimulus of wound healing, which is notably effective in causing hidden tar tumor potentialities to disclose themselves in this way (2), has shown itself to be no less so in the case of those due to benzpyrene and methylcholanthrene.

In reporting on the tumors which appear in response to the non-carcinogenic stimulation of skin tarred some while previously we termed the hidden neoplastic elements responsible for the induced growths latent tumor cells (2). There was the more reason to deem them such because the cells of retrogressed tar papillomas sometimes persist for months afterwards in skin devoid of any trace of the growths, and can readily be induced to form tumors again on non-carcinogenic stimulation (1). Yet

¹ Berenblum and Schoental have just succeeded in demonstrating that tar contains other carcinogens besides benzpyrene (4).

the possibility must be kept in mind that some of the growths called forth by the stimulation of tarred skin may be due to a multiplication of cells which had not completely attained to the neoplastic state before tarring was stopped, this procedure having got matters so well along that mere non-carcinogenic encouragement would do the rest.² Whatever the fact, the hidden cells are, in effect, latent tumor cells, and we will use this term in referring to them.

Materials and Methods

Market-bought, adult gray-brown (agouti) rabbits were employed, as in the work with tar (1). The benzpyrene came from Hoffmann La Roche, Inc., and the methylcholanthrene of the earlier tests from the laboratory of Dr. Louis F. Fieser.³ That used later was procured from the Eastman Kodak Company, and completely purified when necessary by a method kindly outlined by Dr. Fieser. The tar, like that previously employed, came from the Ostergasfabrik of Amsterdam.⁴

The benzpyrene and methylcholanthrene solutions were painted on the entire inner expanse of the ears with a soft brush, twice a week in some cases, thrice in others, and the tar was smeared on, with stripping away of the dried layer prior to every third application. Frequent examinations, chartings, and notes were made to record the incidence, size, character, and situation of the growths induced. Sometimes they were sketched in relation to one another on printed outlines of a standard ear, but usually the shape of the actual ear was traced on a superimposed sheet of cellophane and the tumors drawn in, with subsequent transfer to a card. Individual growths were thus followed over long periods.

To bring wound healing into play holes were punched through the ear with sharp cork borers, 0.4–1.8 cm. in diameter, sterilized by boiling. Many growths were taken by the punch method to learn their character, thus providing additional opportunities to study the effects of healing. Except when large holes had been made near the ear tip, repair was usually perfect, the tissue closing in evenly, without bacterial infection, to form a hyperemic disc. Often as healing progressed tumors appeared, either on the zone of new tissue or next the original edge of the hole. Sometimes the applications of carcinogen were kept up during this period, but with care that none spread to the area of repair. The term *discing* will be used to denote the healing process.

Effects of Benzpyrene in Benzene

No detailed account is available of the effects of benzpyrene on rabbit skin although it has been employed to produce cancers. Rabbits are supposed to be highly refractory to its action (12). Oberling, Guérin, Guérin, and Sannié (13), applying a 1 per cent solution in benzene to the insides of the ears several times a week, noted occasional transitory tumors toward the end of the 3rd month, but Lacassagne and Nyka (14) did not get any until the 5th, while Schürch (15), working with a 0.5 per cent solution, obtained none until the 6th month. Schürch and Winterstein (16), using a 0.3 per cent solution in benzene twice a week as we did, saw the first little "wart" after 198 days and no other until the 237th day. Observers agree that when the applications are kept up for 2 years or more the ears become much thickened and heavily scurfed, and rapidly enlarging growths frequently arise, some having the character

² Experiments dealing with the matter will be described in a later paper.

³ Several grams were made available to us through the generosity of Dr. William H. Woglom of the Department of Cancer Research, Columbia University.

⁴ The tar was the gift of Dr. Karl Landsteiner. The Ostergasfabrik no longer exists.

of benign papillomas while others are frank carcinomas. Brunschwig, Tschetter, and Hamann (17) state that successive crops of benign growths appear first, and that they are frill horns (18) and papillomas, these latter often large, complex, and pedunculated. Klinké (19) mentions only reddening and hyperkeratosis as occurring prior to cancer, which appeared at earliest on the 537th day. He used a 1 per cent solution of benzpyrene in chloroform twice a week.

Course of the Changes.—A solution containing 0.3 per cent of benzpyrene in pure benzene (Merck) was applied twice or thrice a week to both ears of 20 rabbits for from 3 to 15 months. The response varied widely. Occasionally two or three applications resulted in acute inflammation, and further treatment had to be intermitted for a few days. In most instances though, definite changes were evident only after several weeks, the thinnest scurfing with a mild hyperemia. As months passed the mouths of the hair follicles became distended with plugs of keratin, the scurf thickened, and in some regions it became an adherent, fissured, silvery layer, as much as 1 mm. thick, and very tenacious. Where it was thickest the underlying skin was bright pink and slightly raised. Punched out pieces showed, as in benzpyrened mice (20), a differentiating, many-layered epidermis instead of the ordinary covering one or two cells thick. The corium remained compact but came to contain scattered accumulations of macrophages and small round cells. The mouths of the hair follicles became funnel-shaped and they rounded into cysts distended with keratinized material. The sebaceous glands became exceedingly numerous (Fig. 1).

The changes were sometimes almost negligible even after 8 to 10 months⁵ but usually by this time the skin was bright pink under a thick scurf and not infrequently there was slight edema of the corium. The epidermis showed many mitoses and was ruffled or fringed along the base because of irregular extension downwards (Fig. 2), always very slow. Slight scurfing at most took place on the outside of the ear, with thinning of the hair, never such depilation as occurs when the inside is tarred. Occasionally a growth arose, only to disappear later.

Palpation and a lens were employed in the search for tumors. The findings with both ears were nearly alike when they were treated in the same way, but there was much variation from animal to animal. The organs with the most pronounced cutaneous changes usually developed tumors soonest and in greatest number, just as when tarring is done, but exceptions were frequent. The earliest growths were noted after 44 days, as minute, solitary mounds in three instances,⁶ but none appeared in most cases until the 4th or 5th month and sometimes much later. By the end of the 10th month all of the animals had them. When first discovered they were exceedingly small, often like grains of sand or spicules under the palpating finger; and the lens showed them to be tiny horns or scabs or superficial mounds (Fig. 8), these latter usually dry but sometimes fleshy and pink. There were no growths of deeper origin, 4 mm. or more across when they first raised the skin, such as tar often evokes, though occasionally some appeared later on when the local changes were more marked. Many of the growths arose from hair follicles, and first attracted attention as protruding keratinized plugs.

For a long time tumors were few, and they only very gradually increased in number. Most remained so minute as easily to escape notice, and nearly all ultimately disappeared despite the continued application of benzpyrene, though a few gradually enlarged into small cones, low cauliflowers, or filiform horns, almost regularly dry to the base. Very occasionally one grew fast for a few weeks and became fleshy, only to keratinize and flake off later. Some of the small ones were dark gray or black with melanin, as benign tar tumors frequently are when indolent (18).

Character of the Tumors.—Nearly a hundred growths were punched out during the appli-

⁵ Additional pictures of the changes can be found in an accompanying paper (11).

⁶ See Fig. 11 of the accompanying paper (11) for a cross-section of one of the earliest growths.

cations, usually when first perceived. All were epidermal like those procured later, almost all proved benign, and histologically nearly all⁷ were of the same sorts that tarring calls forth, frill horns, papillomas, and carcinomatoids, these latter being papillomas which have taken on temporarily the appearance of squamous cell carcinomas as result of stimulation (18). The growths differed from the tar tumors in certain important respects though. Nearly all were minute, as already stated, often mere tiny, discrete, scab-like keratinizations; and usually they failed to enlarge further. The tar tumors on the other hand are mostly fleshy and often grow rapidly, becoming enormous as the applications are continued.

The microscopic findings will be considered in an accompanying paper.

The character of the tumors justified the conclusion that benzpyrene causes benign neoplastic changes of the same sorts as does tar. What then was the reason for the wide difference in the behavior of the growths? The discing test showed that it was not due to any innate lack of capacity to proliferate on the part of the cells acted upon by benzpyrene.

The healing of punch holes in the benzpyrened ears was often attended by the appearance of tumors. Like the growths developing under similar circumstances on tarred ears they usually appeared soon after repair began, often within 10-14 days, as discrete mound or hassock protrusions situated in the zone of inflammation at the original rim of the hole, or as radial, segmental, or eccentrically placed growths on the new-formed tissue extending toward its center. As a rule they enlarged rapidly until the hole closed and during a few further days, then becoming quiescent and often disappearing as the new disc gradually became scar tissue. Occasionally a growth appeared on the disc soon after it was completed but very rarely later.

The tumors called forth by discing were fleshy, in marked contrast to the tiny mounds and dry scab-like growths elsewhere on the ear, and for a brief while many of them were active, becoming exuberant verrucosities or forming a horn or ulcerating. Yet biopsy showed them to be essentially of the same kinds as those present elsewhere, frill horns, papillomas and carcinomatoids; and when healing was done they took on the same gross aspect as the others, and usually disappeared eventually.⁸ Those that had been carcinomatoids, sometimes very active and perhaps almost a centimeter across, either became papillomas or rounded up into keratinized cysts or vanished wholly, as happens with tar carcinomatoids when no longer stimulated. It was plain that all the tumor cells needed was encouragement.

It seemed possible that the benzpyrene solution might be acting to retard multiplication of the cells it had rendered neoplastic; but later events did not bear out this idea.

As month succeeded month of the treatment the skin became more inflamed and scurfed, some of the existing tumors began to enlarge, new ones appeared more often, and these were not infrequently fleshy and sometimes grew rapidly for a while, occasionally becoming a centimeter across. Yet microscopically they proved to be of the kinds previously noted.

⁷ Occasionally growths developed such as tarring does not elicit, benign adenomas consisting of more or less mature sebaceous gland tissue. They are dealt with in an accompanying paper (11). They had the form of subepidermal mounds 1-3 mm. across, never becoming larger.

⁸ In a previous paper (2) the growths due to the discing of tarred ears have been extensively pictured. The present findings differ in no way from those there portrayed.

The hyperkeratosis and thickening eventually began to approach that pictured in the literature as obtaining at the time when cancer appears, and a carcinoma did develop in one instance. By the 15th month the ears were sometimes twice as thick as the normal and were heavily scurfed. The rabbits remained vigorous throughout and gained weight.

Some of the animals were kept to learn the fate of the tumors after the benzpyrene was left off. This varied with the state of the ears at the time when the applications were discontinued. When they had not undergone much change, reversion toward the normal began within a few weeks,—during which new tumors sometimes arose,—and in proportion as the ordinary aspect was resumed the growths dwindled and vanished, 6 months sometimes elapsing before all were gone. When the changes had been considerable, the ears remained pathological for many months, a few of the old tumors grew slowly and new ones appeared.²

Here was evidence that the benzpyrening produced more and more favorable conditions for neoplastic proliferation as time went on, and that the growths were conditional upon the encouragement thus provided. The nature of this encouragement became evident on microscopic study of the ear tissue. During the early period when the tumors were tiny and inert the corium showed little alteration from the normal and entered almost not at all into the formation of the growths, providing to these a scant stroma at best. But as time went on it became cellular and vascular and cooperated actively with the proliferating epithelial cells, the result being more or less fleshy growths in great part composed of it. These matters are dealt with in an accompanying paper (11). Whether the benzpyrene had any direct influence on the activity of the neoplastic elements themselves is uncertain. But if it had its influence in this respect was overshadowed by the encouragement it provided through the connective tissue changes it brought about.

The Presence of Latent Tumor Cells.—Mention has already been made of the rapid appearance of benzpyrene tumors in response to discing. They arose with such frequency as to indicate that latent neoplastic cells had come into existence in considerable number. Some were present within a few weeks after the benzpyrene treatment was begun.

In an unpublished experiment, carried out in association with Dr. Ian MacKenzie, benzpyrene in benzene was applied six times a week to one ear of 13 rabbits during a period of 27–28 days, and benzene alone to the other ear. The applications were then discontinued and four to six holes, 0.6–1.3 cm. in diameter, were punched in each organ. The discing brought out 6 tumors on the benzpyrened ears of 4 of the rabbits, none appearing elsewhere on the treated surfaces except a single papilloma. The benzened ears remained wholly devoid of growths.

The Effects of Benzene

In appraising the influence of the benzpyrene solution it was necessary to reckon with the effects of benzene as such; for several workers have reported that it gives rise to inflammation, depilation, and freckling of mouse skin when applied for a year or more, although it causes no tumors (21). According to

Stowell and Cramer (22) it brings about thickening and differentiation of rabbit epidermis. Hence we have run tests with it.

Chemically pure benzene (Merck) was painted twice weekly on the inner expanse of both ears of 6 rabbits for periods ranging from 389 to 462 days. The animals were kept apart from those receiving carcinogens. Within little more than a month the skin showed a thin scurfing in some instances, yet after a year the scurf was still slight and branny, though underlain by a pink hyperemia in the most marked instances. The ears were not noticeably thickened. On the 264th day a minute, filiform horn was discovered on one of them, and on the 320th day a very small, dry papilloma on another, of a different animal. Both growths were punched out (Figs. 3 and 4).

From time to time holes were punched in all of the ears but the discing called forth no growths. Sections of the skin procured incidentally showed changes similar to those produced by benzpyrene in benzene, though they came about later and were much slighter. There was the same hyperplasia of the epidermis, with scalloping or irregular fringing along its base, a less considerable distension of the hair follicles with keratin, and a definite increase in sebaceous glands (Fig. 4). The connective tissue showed sparse accumulations of round cells and macrophages.

The benzene not only caused histological changes resembling those due to the benzpyrene solution, but occasionally called forth benign tumors. In both respects, though, it fell far short of this latter, which evidently owed its power to initiate neoplastic change almost entirely to its benzpyrene content.

Some further experiments were carried out to learn how far benzene promotes tumor formation.

Three rabbits were utilized which had benign tumors due to painting the ears with benzpyrene in benzene for 11½ months. When the applications were discontinued one ear of each animal was painted with benzene twice a week for 2 months or 3 months, the other ear serving as control. Scurfing persisted slightly longer where benzene was applied but the tumors of both ears dwindled and disappeared at about the same rate.

Benzene was painted on one ear of 6 normal rabbits twice a week for 2 months and benzpyrene in benzene on the other, after which both were treated with the benzpyrene solution. The skin which got it for the longer period developed growths sooner and in larger number, the difference approximating that due to a 2 months' difference in the period of benzpyrene applications, as evidenced by the records of other rabbits carried along at the same time.

The promoting effect of benzene on tumor formation is evidently almost^t negligible; yet since the growths it induced were of the kinds which require encouragement if they are to appear at all one cannot write off its influence entirely.

The Effects of Benzpyrene in Mineral Oil

So gradually did benzpyrene promote tumor growth when dissolved in benzene as to suggest the possibility that a medium might be found in which it would have no influence of the sort. Mineral oil was tested because Twort and Twort (6) have reported that carcinogenic substances when dissolved in it are rela-

tively ineffective in producing skin tumors in mice. The fact that both ears of the individual rabbit respond with nearly the same number of growths when treated alike rendered possible a direct comparison of the influence of oil and benzene as mediums for the carcinogen.

One ear of 12 animals was painted twice weekly with a 0.3 per cent solution of benzpyrene in benzene and the other with the same amount in mineral oil (heavy Californian liquid petrolatum, Squibb). The applications were kept up for 10-15 months. The solution in benzene gradually caused the skin to become thinly scurfed, slightly raised, and reddened. The oily solution on the other hand had no evident effects for a long while; but after 200 to 300 days an enlargement of the hair follicles could be perceived in most instances, though often so slight as to be discerned only when normal ears were placed next the altered organ. The enlargement was general (Fig. 6), and gradually became marked in some instances, especially near the middle of the ears where all the follicle mouths widened to nearly a millimeter across. The skin between them retained its normal pearly luster.

The animals had no tumors when examined on the 73rd day and were not looked at again until the 175th day. Seven of the ears receiving benzpyrene in benzene were then found to have 1 to 5 growths of the usual benign sorts, and an eighth had 14, whereas the ears getting the carcinogen in oil were still bare of them (Chart 1). By the 231st day all of the ears painted with the benzene solution carried tumors, whereas only one receiving the oily solution had any. This one bore a solitary growth, the animal (D.R. 3-59) being exceptionally responsive to the carcinogen, as indicated by the presence of 16 tumors where it had been applied in benzene. On the 297th day growths were still absent from the oiled ears of 9 of the surviving 11 animals. Tarring was now begun on four of the ears that had received the benzene solution, and this brought the test to a close in these instances, the rabbits soon transferring some tar to the oiled organs with result in scurfing, inflammation, and rapidly growing tumors in 3 cases. Two of the 7 animals in which the original applications were kept up still had no tumors on the oiled ears on the 346th day.

The differences in the effects of the solutions continued to be pronounced up to the time when the experiment was ended. With the exception of a carcinoma, to be described further on, the tumors that arose on the skin treated with benzpyrene in oil were minute mounds, or hair follicles exceptionally distended by keratinized plugs. They failed to keratinize and become scab-like as did the growths on the ears receiving the benzene solution, and most of them remained less than 1 mm. across. Some disappeared, others were punched out, and a few eventually reached a diameter of 3-4 mm. Those removed were of the ordinary benign sorts—papillomas, frill horns, benign sebaceous adenomas, the carcinoma already mentioned, and an unerupted carcinomatoid (or perhaps a carcinoma), which was taken *in toto* and found to be doing badly amidst round cells (Fig. 5). The growths produced by benzpyrene in benzene were of the same types but had more tendency to enlarge.

These results accord with the finding in mice, that benzpyrene is relatively ineffective in producing tumors when dissolved in mineral oil. This might have been because the solution had little power to initiate neoplastic change or because it failed to promote the multiplication of tumor cells, or both. Discing tests showed the results to be wholly due to failure of the oily solution to encourage cell multiplication.

Five rabbits were utilized of the group just described. On the 411th day of the paintings with benzpyrene in oil and benzene respectively, four holes 5-6 mm. in diameter were punched

Influence of the Solvent on Tumor Formation
(Mineral oil and benzene as solvents of benzpyrene)

Rabbit No.	Solvent	Days	231	297	346	408	436	456	456 days
Tumors on general surface of ear									
3-54 { Oil					2		1 K		
3-54 { Benzene			1	1	2	5			
3-50 { Oil						2			
3-50 { Benzene			3	6	5	6	3 K		
3-59 { Oil						1 †			
3-59 { Benzene			14	16					
Tumors on healing area									
3-58 { Oil					1		0 P		
3-58 { Benzene			2	1	2	7	13	7	
3-53 { Oil							1 ^Δ	3 ^Δ 6 ^Δ	
3-53 { Benzene			2	9	2 ^Δ	9	11	12	
3-60 { Oil							1	10	
3-60 { Benzene			3	3	9	7	7	9	
3-51 { Oil							4(4) P*	0 2	
3-51 { Benzene			1	2	2	6	12(5)	5 5	
3-55 { Oil							1	1 P	
3-55 { Benzene			1	2	2	1	1	1 4	
3-61 { Oil							3		
3-61 { Benzene			5	8	7 T	18			
3-56 { Oil							2		
3-56 { Benzene			2	12	20 T	17	Discarded		
3-52 { Oil							0		
3-52 { Benzene			2	4	5(2)	11			
3-57 { Oil							14 T		
3-57 { Benzene			13	27 T	17				

CHART 1. *Influence of the Solvent on Tumor Formation: Mineral Oil and Benzene As Solvents of Benzpyrene.*—For analysis of the findings see text. The heavy horizontal lines mean that tumors were absent during the periods over which they extend. P* = holes punched in the ears on the 411th day. P = holes punched on the 436th day. The numbers in brackets = number of tumors removed incidentally to the punching of the holes. Δ = one of the growths is a carcinoma. † = died of intercurrent causes. K = killed.

Each of the broken line circles shows the original margin of a punch hole, and the inner, solid line circle shows the size to which it had diminished when the charting was done. The tumors elicited by the healing are blacked in to size. The numbers set down in the column for the 456th day tell how many tumors were then present where no holes had been made. × 1.

in each ear of one of the animals (D.R. 3-51) at approximately corresponding situations, and on the 436th day three holes 5-9 mm. across were made in each ear of the other 4 individuals, also at corresponding spots and of approximately equal size. Some growths already present were removed for study incidentally. The areas of healing were avoided at the later paintings of the ears.

Chart 1 shows that 2 of the 5 rabbits tested had been the last of the 12 animals to develop tumors on the oiled ears. In the case of a third individual (D.R. 3-58) 2 growths had arisen some weeks prior to the punching but they had vanished and the ear bore none when it was done. The 4 tiny growths on the oiled ear of D.R. 3-51 were all punched out at this time, as were also 5 of the 12 existing on the ear that had been painted with the benzene solution. Taking the group of rabbits as a whole, this latter had caused growths 66 to 233 days before the oily solution produced any and had elicited them in fair number on four ears by the time the test was begun. Every circumstance seemed to favor the supposition that discing would call forth many more tumors from the skin painted with benzpyrene in benzene, even to the size of the holes which were somewhat larger than those in the oiled skin, furnishing a greater total length of raw edge from which healing could occur and hence providing proportionately more opportunity for tumors to arise.

Healing took place at approximately the same rate on both ears of each rabbit but with thicker discs of new tissues where the benzpyrene in benzene had been applied; and soon after it began one or more tumors appeared at the edge of the holes or upon some of the forming discs and rapidly enlarged into discrete, fleshy hemispheres or hassocks or mounds (Fig. 6), which often extended in radially or segmentally with the new tissue. Three of the animals were killed after only 20 days of discing, to obtain sections of the tumors while actively growing. The holes were then but partly closed and complete closure in the case of the other animals was attended by no new tumors. Chart 1 makes clear that the reparative process regularly elicited more growths from the skin which had received benzpyrene in oil, and they were generally larger.

Of 10 disc tumors, which were examined microscopically, 2 were frill horns, 4 papillomas, and 4 had the microscopic appearance of carcinomas but in view of the rarity of such growths were almost certainly carcinomatoids. The largest of all (D.R. 3-51, Fig. 6),—which had arisen on an ear treated with benzpyrene in oil,—was indubitably a carcinomatoid, as its later history proved. On the 16th day of wound healing it was a discrete, elongate mound 4 mm. high, ulcerated in its more recent portion; and a thin, sagittal slice removed next day showed (Fig. 7) what appeared to be active, squamous cell carcinomatosis with differentiation into benign papillomatosis in its older region. No infection followed, the cleft closed smoothly, and the entire growth became a papilloma later and eventually regressed.

Sections disclosed the fact that the solution of benzpyrene in mineral oil had changed the skin more than its gross aspect suggested. Its surface, though appearing smooth, was actually covered with a thin sheet of keratinized cells; the living epidermis had become many-layered and differentiating; the hair follicles were funnel-shaped; the sebaceous glands had increased greatly in number; and there were more macrophages and lymphocytes in the corium than ordinarily. In sum, the changes resembled those that benzpyrene in benzene had brought about on the other ears of the same animals, but they were far less considerable and there was no gross thickening such as these organs had undergone.

The experiment proved that the solution of benzpyrene in mineral oil had a capacity to initiate neoplastic change equaling if not exceeding that of the

carcinogen in benzene solution⁹ but that it had almost none of the power of this latter to promote tumor formation. Its shortcoming in such respect was manifestly responsible for its relatively slight carcinogenicity, as judged by the usual criterion of growths induced to appear.

The Effects of Methylcholanthrene

The carcinogenic effects of methylcholanthrene were next studied. No tests with it on the skin of the domestic rabbit have been found in the literature.

A 0.3 per cent solution of methylcholanthrene in benzene was painted twice weekly on the ears of 9 rabbits. The same cutaneous changes ensued as with benzpyrene in benzene, but they appeared earlier and soon became more considerable, a branny or snake skin scurf forming in some cases within 2 weeks and thickening during the next 2 or 3 months into a rough, fissured, tenacious layer of silvery keratin. The surface beneath was bright pink and slightly raised, occasionally as much as a millimeter. One animal had a minute growth by the 15th day of the applications, another had one on the 17th, and several developed them shortly afterwards. The number of tumors increased faster than on benzpyrened skin, though much more slowly than on tarred. While the majority of the growths remained dry and minute, as with benzpyrene, some grew steadily from the first, as squat cones, peaks, or cylinders (Fig. 8), sometimes with bulging bases, or as fleshy, hemispherical mounds which later erupted and became cauliflower papillomas, usually keratinizing and dry, and never more than a few millimeters across.

As months passed the inflammation and thickening became more pronounced than in the case of benzpyrene, the tumors much more numerous, and a larger proportion of them grew and became fleshy. None exceeded a centimeter in diameter however, though the applications were kept up, and even the largest growths keratinized and vanished eventually. In some instances the methylcholanthrene was stopped soon after the first growths appeared, and then, if the applications had not been long kept up, the skin began to revert to the normal, the tumors disappearing as it did so, just as happens under similar circumstances after tarring or benzpyrene. When on the other hand treatment had gone on for more than 4 months the skin remained pathological for as long as 3 years and not only did a few of the tumors persist but many new ones arose.²

Microscopically the changes were like those due to benzpyrene in benzene but more considerable. Pieces of skin punched out after the applications had gone on for 6 weeks to 3 months often showed minute, discrete necroses in the midst of a many-layered, differentiating epidermis. They were usually situated next the widened mouths of hair follicles, often at the angle of junction with the surface epithelium (Fig. 9). Here because of cupping there was a special opportunity for the solution to be retained. Occasionally the epidermis at the affected spots became necrotic throughout its thickness, but ordinarily the Malpighian layer was primarily affected, the basal elements remaining alive, and those near to the surface in specimens procured early. Fluid gathered secondarily amidst the epithelium, with result in lenticular or football-shaped accumulations too small to be seen in the gross; polymorphonuclear

⁹ Mineral oil penetrates poorly into rabbit skin as compared with other oils (23), and one might suppose that benzpyrene dissolved in it would not reach the epidermal cells easily. But as against this disadvantage the treated skin remains nearly bare of shielding scurf, and the oil keeps the carcinogen dissolved,—as witness the brilliant purple fluorescence to be seen in ultraviolet light. Much of the benzpyrene applied in benzene falls out in powder, as the light test also shows.

leukocytes wandered in; and there was sometimes ecchymosis in the corium just beneath. We have occasionally come upon similar blisters,—for such they are,—in skin painted only with benzene; and benzpyrene dissolved in benzene not infrequently gave rise to them, though none have been encountered where it was applied in oil. But methylcholanthrene in benzene produced them most often. Later, as the applications of it were kept up, no more occurred. We have reported somewhat similar epidermal necroses in mouse skin submitted to methylcholanthrene in benzene (2).

Character of the Tumors.—Many of the tumors were punched out when first noted. They were of the same benign sorts as those evoked by benzpyrene, though often relatively fleshy because of a larger connective tissue component, as were nearly all of those removed later. Now and then a recently appeared mound was noted to be hemorrhagic, and several of these were excised and found to resemble anaplastic carcinomas (see Fig. 6 of the accompanying paper (11)). But since the hemorrhagic mounds of identical appearance which were left undisturbed remained in general small and became papillomatous, or rounded up into cysts or disappeared, they must be put down as mostly mere carcinomatoids. Very occasionally one grew and proved to be a cancer, as described further on.

Methylcholanthrene in benzene was definitely more effective than benzpyrene in producing visible tumors, which appeared earlier,—in two animals by the 15th and 17th days, respectively,—and tended to be more vigorous. It caused more hyperplasia and hyperemia of the skin, changes known to favor the early appearance and rapid enlargement of tumors. Also it was superior to benzpyrene in initiating latent neoplastic changes as discing tests showed.

Twenty-three holes 5–10 mm. across were punched in the ears of 8 of the rabbits after they had been painted with methylcholanthrene for from 53 days to 3 months. The healing called forth 17 growths in 6 animals. The healing of 16 comparable holes in the ears of 6 animals which had received benzpyrene in benzene for much longer periods, from 4 to 18 months, called forth only 7 tumors, in 4 animals.

A subsidiary experiment was carried out to learn whether, when many ears were treated in the same way, the number of visible growths arising gave any indication of the relative number of latent tumor cells present. This proved to be the case.

The ears of a special group of 7 rabbits were painted on the inside twice a week for 108 days with methylcholanthrene in benzene and 2 days after the final application four holes, approximately 9 mm. across, were punched in each of them at nearly the same spots from animal to animal. Only a few of the rabbits had growths at the time but they appeared on others later. As Table I shows, there was a rough correlation between the number of tumors that discing called forth and the number present elsewhere on the ears.

In rabbit 1-03, 2 of the 4 growths which had formed by the 17th day were fleshy papillomas already 6 and 7 mm. across. No attempt has been made to learn how soon methylcholanthrene can bring about neoplastic change but it must have done so very rapidly in this instance. The cell multiplication which gave rise to such large growths can scarcely have taken place overnight.

Effects of Methylcholanthrene in Ether and Mineral Oil

Crabtree has found benzpyrene to be notably carcinogenic for mouse skin when dissolved in ethyl ether containing 2 per cent of mineral oil (24). We have applied a 0.3 per cent solution of methylcholanthrene in Crabtree's solvent (U.S.P. ingredients) to the ears of 4 rabbits twice a week for 8 months. Scurfing, superficial thickening, and inflammation came about as quickly as

TABLE I
Visible Growths As Indicating Latent Tumor Potentialities in Skin Painted with Methylcholanthrene in Benzene

Rabbit No.	Situation of the tumors	Day . . 17	52	109	110	144	185
1-01	Entire ears	2	↑ 108th day—Methylcholanthrene stopped ↓	6	4 holes punched in each ear Total healing edge for each animal 24.4 cm.		
	Undisturbed areas					13	11
	Disc areas					4	4
1-02	Entire ears	2		5			
	Undisturbed areas					13	12
	Disc areas					2	3
1-03	Entire ears	4*		4			
	Undisturbed areas					5	4
	Disc areas					9	9
1-05	Entire ears						
	Undisturbed areas					4	4
	Disc areas					1	1
1-99	Entire ears						
	Undisturbed areas					2	1
	Disc areas					2	0
1-00	Entire ears						
	Undisturbed areas					1	1
	Disc areas					1	1
1-04	Entire ears						
	Undisturbed areas						
	Disc areas						

* 2 of the growths were 6 and 7 mm. in diameter respectively.

when the carcinogen was dissolved in benzene, though the scurfing was less pronounced; and tumors arose at about the same rate. They were of the usual benign sorts. After the applications were discontinued the skin changes persisted and new tumors continued to appear for a long time.² Subsidiary tests of the ether-oil solvent as such showed that it caused only the faintest scurfing of the skin after many months.

Relative Effectiveness of the Hydrocarbons in Producing Cancer

Previous workers have applied benzpyrene to rabbit ears until cancer arose,—by which time the skin was greatly changed as a rule and carried large benign growths. We have left off applying it long before this state of affairs had been reached, yet have obtained two presumptive carcinomas. The general finding has been that cancer develops only after 1 to 3 years, but Lacassagne and Nyka (14) obtained it within 6 months in one animal, painted with 0.1–0.5 per cent of the hydrocarbon in benzene. We had what may have been a similar instance.

The ears of D.R. 74 had been painted for 194 days with 0.3 per cent benzpyrene in benzene when a rapidly growing, fleshy mound was first noted which soon became a raised, ulcerated disc. At that time 4 other tumors were present, minute and of the usual benign sorts. A piece punched from the ear to include the enlarging growth showed what appeared to be a squamous cell carcinoma. Within the next month it reached a diameter of nearly 2 cm., was ulcerating and weeping, and had extended to the outside of the ear through the biopsy hole. Then the animal died of other causes.

This rabbit provided the only malignant tumor occurring in 20 animals receiving 0.3 per cent benzpyrene in benzene on one or both ears, 11 of them for periods of from $11\frac{1}{2}$ to $15\frac{1}{2}$ months, the others for from $8\frac{1}{2}$ to $11\frac{1}{2}$ months. And even the character of this growth is dubious. For the benzpyrene had caused the skin to become unusually inflamed and scurfy, and the tumor had been subjected to the stimulus of discing, conditions both which act to encourage carcinomatoids.

One of the rabbits receiving benzpyrene in mineral oil on one ear and in benzene on the other (D.R. 3-53 of Chart 1) had on the 346th day a raised, raw disc 4 mm. across, under the recurved edge of the oiled ear, where it may have been present for several weeks undiscovered. A piece punched from it showed what appeared to be a very anaplastic, squamous cell carcinoma with oat-shaped cells. The benzpyrene applications were stopped on the 459th day. Thereafter the disc grew, although the skin round about appeared normal save for slight enlargement of the hair follicles; and it was still progressing when the animal was killed on the 548th day. At this time it was 3 cm. across and had penetrated to the outside of the ear, with result in a raised ulcer 2 cm. in diameter. Microscopically it was now an ordinary, moderately anaplastic, squamous cell carcinoma. There were no metastases. When it was first noted only one other tumor was present on the ear,—though the wound healing test, as applied shortly afterwards, showed latent neoplastic cells to be numerous (Chart 1, D.R. 3-53); but by the time the carcinogen was left off 6 minute benign growths had arisen, none appearing later.

Methylcholanthrene in benzene induced cancer much sooner and oftener than benzpyrene, carcinomas developing in 3 of 9 rabbits to which it was applied.

In one animal the cancer became visible after only 12 weeks. Several minute papillomas and frill horns were already present. The tumor appeared as a hemorrhagic mound which rapidly extended in the long axis of the ear and soon ulcerated. When it was 3.1 cm. long, 1.3 cm. broad, and 8 mm. high (Fig. 10), a piece was punched out of it which showed what looked like squamous cell carcinomatosis. During the next few weeks the tumor disappeared almost entirely, although the hole healed smoothly and the methylcholanthrene applications were kept up: at one time a scarcely raised, raw area 1.3 cm. long and 2 mm. across alone remained. But by the time the carcinogen was stopped the growth had begun to enlarge again, $2\frac{3}{4}$ months after it was first discovered, and during the succeeding 5 months

it involved more than half of the ear in a thick, fleshy, ulcerating mass, and metastasized to a basal lymph node. Pieces of this metastasis were implanted in the muscles of the legs at six situations, where they soon gave rise to large nodules of squamous cell carcinomatosis.

In another rabbit the methylcholanthrene had caused marked scurfing and inflammatory thickening by the time it was discontinued, after 8 months, and scattered, minute growths were present then, with several dry cauliflowers which had reached a diameter of 3-4 mm. During the succeeding 2 weeks, before any involutionary changes were evident, a purple, sanguineous, subepidermal mound developed on one of the ears and during the next 2 months it became an ulcerated disc 2 cm. across; yet it was only 2.5 cm. across after half a year more. Toward the end of this time a neighboring, small, cauliflower growth, previously dry but kept wet of late by the foul, thin fluid from the ulcer, began to grow rapidly and became a raised, ulcerated disc 7 mm. in diameter, with slanting edges. The other tumors on the ear had all remained minute and dry. A large piece of tissue was now punched out to include a part of both the ulcerated growths, and microscopically this showed what appeared to be two different kinds of squamous cell carcinomatosis. The hole closed smoothly yet neither tumor seemed stimulated but on the contrary the broad ulceration of the bigger of the two growths healed over completely and the growth became a fleshy hemisphere covered with epidermis, though of the same diameter as before. Soon afterwards the smaller tumor, now dry again, flaked off and disappeared. Another punch biopsy, small this time, to learn the microscopic character of the hemisphere, showed that it consisted mostly of new formed connective tissue of myxomatous aspect with scattered nests of anaplastic epithelial cells. Again the discing took a smooth course and again it failed to encourage the tumor, which dwindled and in another 2 months was gone. Elsewhere on the ear,—which was still markedly pathological,—new, minute dry growths had come in, and there were 25 of them now as compared with 12 when the presumptive carcinoma first arose. Two years have elapsed since this latter disappeared and it has not recurred although a hole was punched through part of the area it once occupied, with the aim of stimulating any of its cells which might have survived. The ear now carries 59 other growths.

The history of the tumor which behaved as if malignant while bathed with foul, irritant fluid resembles that of tar papillomas under like conditions (1) and shows it to have been a mere carcinomatoid.

In a third rabbit what appeared to be a cancer was first noted 267 days after methylcholanthrene was left off, after an application period of 215 days. The ear was still markedly pathological when it arose, and carried 14 small benign growths, as compared with 10 when the carcinogen was stopped. The tumor had taken origin from a tiny, scabbed growth present 4 months and it was now a raised, sanguineous disc, 8 mm. across, with a weeping, foul, pultaceous surface. The disc flourished only briefly: in 6 weeks it was gone, leaving behind a scar amidst which were a few minute, keratinized cysts. No recurrence has appeared in the succeeding 2 years and 4 months, though there are now 69 small, dry tumors elsewhere on the ear. The healing of a hole made to include part of the spot where the growth once existed has failed to call it forth.

The possibility must be considered that some of the tumors here classed as cancers were carcinomatoids. But as already stated the growths of this sort evoked by tarring can maintain themselves only if aided, altering to papillomas or rounding up into keratinized cysts or disappearing entirely when the skin changes favoring them have worn off. None occur under ordinary conditions after tarring is stopped (1). The small and relatively inert carcinomatoids elicited by benzpyrene and methylcholanthrene in the experiments now de-

scribed have regularly undergone a similar regression when no longer aided. The growths with the morphology of carcinomas which are now under discussion arose when the conditions were all against carcinomatoid manifestations.

Non-Specific Nature of the Tumor-Promoting Influence of the Hydrocarbons

The power to encourage tumor formation and growth, which is exhibited very strikingly by tar, and of which methylcholanthrene has little and benzpyrene less, is not peculiar to the carcinogens,—a fact sufficiently attested by the results of discing. Repeated paintings with a turpentine-acetone mixture

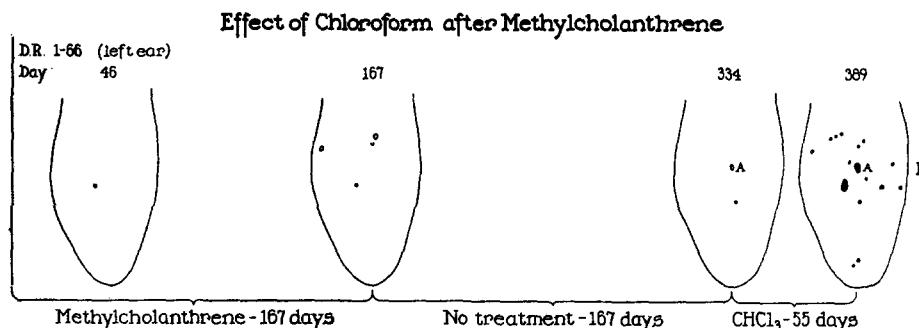


CHART 2. Chloroform was dropped into the external auditory canal twice a week, beginning 167 days after the last methylcholanthrene, when the ear was almost free from growths. It spread to the inner surface of the organ and there the skin soon became swollen and ruddy, tumors which had long since vanished reappearing and new ones arising as well. Three of them ulcerated (charted in black). The animal was now killed and the ulcerated growths were found to have the morphology of invasive carcinomas, as is true of carcinomatoids subjected to stimulation. One of them (A) had been present for several months prior to the chloroform applications, as a dry, gray, cauliflower papilloma. The tumors represented in outline were all of the latter sort or frill horns.

sometimes lead benign tumors to appear on skin previously tarred, and this may happen also when it is bathed in the irritating fluid from an ulcerated cancer (1). During experiments on the influence of methylcholanthrene to hasten the derivation of cancers from virus papillomas (25) we have repeatedly encountered instances of the latter sort. One will be given.

The papilloma virus (Shope) was tattooed into twelve widely separated spots on each side of a large domestic rabbit, and 7 days later nine of the spots, with a zone of skin around each of them, were painted with a 0.3 per cent solution of methylcholanthrene in Crabtree's fluid. The applications were repeated three times a week for slightly more than 4 months. Virus papillomas appeared promptly at every inoculation site and the skin round about them became thickened and scurfy and showed a few, minute, dry growths such as methylcholanthrene usually elicits. After the treatments were left off it involuted and within 2½ months had resumed the normal aspect, the growths nearly all disappearing. Cancers now arose from some of the treated virus papillomas high on the sides (25), ulcerating and destroying them, and

soon the skin further down, including some zones that had been methylcholanthrened, was constantly wet with foul exudate and became thickened and inflamed. On these zones but on none of the others previously treated with methylcholanthrene papillomas and frill horns such as this carcinogen induces appeared and enlarged rapidly.

Chloroform has a marked effect to cause latent neoplastic cells to form tumors, as we discovered by accident.

Occasionally the external auditory canal of ears long previously painted with methylcholanthrene and still carrying growths became infested with mites. To kill them chloroform (U.S.P.) was dropped into the canal, and in several instances, through a technician's error, it was used for nearly 2 months and allowed to spread on the surface of the ear. There the skin became swollen and pink, and many additional tumors arose and grew rapidly, some of them having the carcinomatoid form. Chart 2 provides an instance in point.

Chloroform was applied very lightly thrice a week to one ear of a group of Dutch belted rabbits which had been painted on both ears some while previously with 0.3 per cent methylcholanthrene in Crabtree's fluid throughout a period of 5 months. No acute inflammation resulted but a gradual scurfing; and in some cases many small new tumors appeared as not on the control ears (Chart 3).

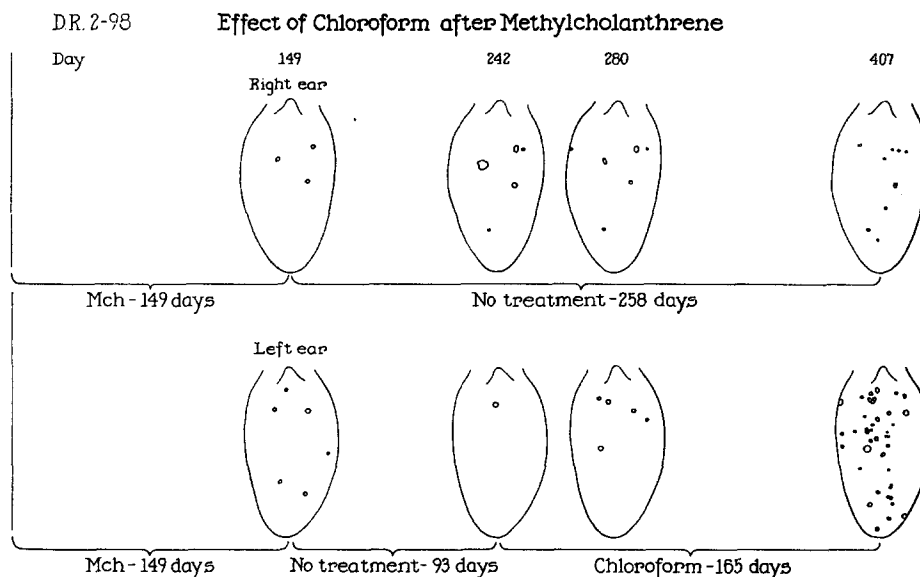


CHART 3. Chloroform applications were begun 93 days after the last treatment with methylcholanthrene. It was lightly painted on the inner surface of the left ear, which carried a single residual growth, and the applications were kept up for 165 days. At the end of this time numerous papillomas and frill horns had appeared as compared with relatively few on the control ear.

Comparison with the Effects of Tarring

The effects of tar have been detailed in previous papers (18, 1). They differed significantly from those of benzpyrene and methylcholanthrene.

Two or three tarrings caused acute inflammation, with swelling, depilation, and scurfing or maceration under the tar layer. Later the edema disappeared, the swelling though less became firmer owing to connective tissue proliferation, and there was great distention of the hair follicles with keratin. Often growths appeared early, after less than 2 weeks in some cases. Many grew fast, and became huge, fleshy, complicated in structure, and not infrequently pedunculated. Their activity was traced to the favoring local conditions brought about by the tar, notably the excited, hyperemic state of the corium (1).

In contrast with these findings a long time passed before benzpyrene produced any considerable cutaneous changes, and then they were superficial. Methylcholanthrene while more effective was far less so than tar. No tumors due to benzpyrene appeared until the 7th to 8th week at earliest, and they nearly always disappeared later or remained tiny though the applications were kept up. The growths caused by methylcholanthrene arose sooner and had more vigor but also tended to vanish or remain small. In both cases most of the persisting tumors took the form of dry cauliflowers or narrow horns, scarcely raised plaques or rugosities, or saucer depressions,—to be described in an accompanying paper (11). The contrast with the florid excrescences called forth by tarring was extreme. Only after many applications did the skin become markedly inflamed, and some of the tumors enlarge progressively and become fleshy.

The influence of tar extended well below the surface, many of the growths it elicited being subepidermal mounds 3–4 mm. across when first perceived, in consequence of proliferation in the depths. The early benzpyrene tumors were, by contrast, always superficial affairs and nearly always minute, and so too with the majority of those due to methylcholanthrene though occasionally they arose as mounds of deep origin.

The various tars we tested all caused emaciation, liver cirrhosis, ascites, and death when applied for more than a few months, and one cannot tell to what state they would have brought the skin if they had been applied for as long as was necessary for the production of tumors with benzpyrene. There was no such difficulty with methylcholanthrene, which evoked tumors almost as quickly as tar in some instances though it failed to encourage their subsequent growth to anything like the same extent. Both agents initiated many hidden neoplastic changes, as the discing test showed, and benzpyrene did this also though more slowly.

Tar evoked many more carcinomatoids than the pure hydrocarbons and they were more aggressive owing to the stimulation it provided. Yet it only infrequently gave rise to cancers, 12 in all thus far in nearly 300 rabbits, of which not a few were tarred for two or three periods of several months each, with intervals between. In our limited experience methylcholanthrene has far outstripped it as a carcinogen in the proper sense of the term. Yet the cancers methylcholanthrene caused were hesitant, retrogressed, or at most were slow-growing, whereas all but two of those due to tarring grew fast. Here as in the case of the benign tumors the indirect, promoting influence of tar seems to have come into play, the cancers having arisen on ears which were inflamed and highly vascular, either because tarring was still kept up or because the many growths it had already called forth were crowded and macerating. The scurfy, dry, methylcholanthrened skin was by comparison much less favorable to them.

When tarring was discontinued after it had elicited many tumors the skin resumed the normal aspect months or even years sooner than that on which growths had appeared in response to methylcholanthrene, and though for a while growths might still arise they all eventually dwindled and vanished (1) save such as had large connective tissue cores, these

becoming fleshy tags. The skin receiving methylcholanthrene on the other hand often remained pathological for several later years, and though most of the benign tumors sooner or later vanished new ones kept on appearing, with result in an increasing horde.² Some of the rabbits of which this was true are still under observation, and the most recent findings suggest that the tumors will all eventually disappear, if only the animals live long enough.

In sum, the very considerable differences in the ability of tar, benzpyrene, and methylcholanthrene to produce tumors are largely due to differences in the ability to promote neoplastic growth.

DISCUSSION

The experiments show that by utilizing the benign, conditional tumors of rabbit skin as test objects and the stimulus of wound healing to bring out the presence of latent tumor cells it is possible to distinguish between the initiating and promoting effects of chemical carcinogens. The initiating effect can be observed almost by itself when benzpyrene is applied in mineral oil and ether. Numerous cells become neoplastic then, yet so little is their multiplication encouraged that for a long while the skin may carry few visible growths or none. The other carcinogenic solutions we tested had all a promoting effect which was important in determining the appearance of tumors.

The current lack of discrimination between the initiating and promoting elements in carcinogenesis finds expression in terms such as "tumor induction," which includes them both, and also in the criteria employed in rating the relative effectiveness of the carcinogens. A standard often used is the elapsed time before the first papilloma appears, but some workers take instead the time before the first progressively enlarging papilloma, while others do not class a growth as a tumor until it has exceeded an arbitrarily set diameter, and others yet have an eye only to the first carcinoma. Manifestly the real criterion is the time required to render the first cell neoplastic; for oncogenicity properly speaking is contained in that act. To perceive when it takes place is impossible, needless to say; yet by forcing latent tumor cells to disclose themselves one can come close to the event.

Nature of the Promoting Influence.—Our observations indicate that almost anything which induces hyperplasia,—turpentine, irritant secretions, trauma, chloroform,—may cause growths to start forth if latent tumor cells have come to be present in the epidermis. The neoplastic response to wound repair exceeds even that to continued tarring and is vastly greater than that to benzpyrene or methylcholanthrene. We have analyzed the encouraging factors in a previous paper on the action of tar (1). It is conceivable that some carcinogens stimulate the epithelium directly.

At first sight the observation that solutions of methylcholanthrene and benzpyrene promote the formation of tumors,—if only ever so little when the benzpyrene is applied in mineral oil,—seems to contradict the finding that many of the polycyclic hydrocarbons, benzpyrene and methylcholanthrene

amongst them, may retard neoplastic proliferation (9, 10). These substances behave as protoplasmic poisons when present in concentration (26), and blistering may be caused by solutions of the strength ordinarily employed for carcinogenic purposes as we have shown. The cells of rabbit tumors directly painted with such solutions not infrequently become vacuolated and unhealthy as we have also observed. Previous workers have commented upon the fact that tumors due to the polycyclic hydrocarbons frequently take origin at some distance from where they are applied, not at the site of greatest concentration, necrosis occurring there (26, 27). All of this is to say that in excessive quantity they cause injury. Yet it is equally certain that in small amounts they bring about tissue proliferation, as a secondary consequence perhaps of harm sustained, and that where this happens tumors are most likely to arise. The slowly increasing chronic inflammation of the skin of rabbits treated with benzpyrene and methylcholanthrene favors neoplastic growth to an extent which more than compensates for any directly repressive effect of the carcinogens.

The Adjuvant Promoting Influence of Solvents.—The influence of solvents on the response to carcinogenic substances is generally attributed to a greater or less ability of these fluids to enable the carcinogen to reach cells of the susceptible sorts and to maintain it in contact with them. But there is more to the matter. A solvent may be itself a promoting agent and may enhance the promoting influence of the carcinogen dissolved in it. Benzene applied as such to rabbit skin sets up a definite if almost negligible tissue disturbance of the kind favoring tumor formation; and benzpyrene in benzene is much more effective as a promoting agent than when it is in mineral oil,—although under these latter conditions it initiates as many neoplastic changes. The difference, measured in terms of the gross tumor yield, is great.¹⁰

Relative Ability of the Carcinogens to Initiate Neoplastic Change.—To judge from the results of discing, tar seems to equal if not to surpass methylcholanthrene in the rapidity with which it brings about neoplastic changes in rabbit skin. But this may be because it disorders the tissue more rapidly and deeply, with result that its carcinogenic component has greater opportunities, reaching more cells,—a fact clearly evident in the frequency with which tar tumors appear from out of the depths of the cutis. Benzpyrene is relatively ineffective as an initiating agent.¹¹

¹⁰ Stowell and Cramer (22) have lately concluded that the hyperemia of rabbit skin which follows upon the repeated application of methylcholanthrene in benzene is consequent on a combined effect of the carcinogen and its solvent. They did not study tumor production.

¹¹ Of late the term *cocarcinogenic* has been applied to agents which hurry the appearance of tumors in response to carcinogens. The prefix *co-* is unfortunate as implying that such agents have themselves the power to induce neoplastic change,—that when they are used two carcinogens are working together. This is not the case. The papers dealing with the “cocarcinogens” show clearly that the substances thus designated do not cause neoplastic changes but act either by enabling the real carcinogens to reach susceptible cells or by promoting the

Non-Specific Promotion.—It seems certain that many agents and influences which have no actual carcinogenicity will be found to stimulate the multiplication of latent neoplastic cells. The growth of clinical tumors is known to be encouraged by a great variety of intercurrent factors and there would seem to be no reason why many of these should not prove similarly effective upon hidden individual cells or groups of cells. The observation that chloroform can cause latent tumor cells to form growths means that caution must be used in the interpretation of data obtained when it is employed as the solvent of carcinogens or in tests for the adjuvant or summing effects of other substances (*e.g.* estrogens dissolved in chloroform). When such substances appear to hasten carcinogenesis, it may be merely that the solvent is promoting proliferation. Whenever one carcinogen appears to increase the effects of another the findings must be scrutinized to learn whether it has actually furthered the initiation of neoplastic change or has merely hastened the multiplication of elements already in the neoplastic state,—this as distinct from its carcinogenic power.

The Promoting Influence As a Factor in the Production of Mouse Tumors.—The proliferation of neoplastic cells plays an important part in the genesis of mouse tumors.

Deelman's finding (28), long discredited, that the tar tumors of mice are prone to arise where skin is healing, has lately been confirmed in rabbits (2) and still more recently in mice (29). Some of the growths that healing calls forth in the latter animals are cancers. Mider and Morton (30) have reported that mouse papillomas elicited by a single cutaneous application of methylcholanthrene in benzene disappear promptly or shrivel and drop off, whereas growths of the same kind that follow upon repeated painting tend to enlarge and often become malignant. They concluded that the differences must be due to local changes induced by the carcinogen.

The share of the promoting factor in the production of tumors is less readily discerned and estimated in the mouse than in the rabbit. A main reason is that mouse skin rapidly becomes disordered even by benzpyrene. A single application of this substance or of methylcholanthrene in benzene renders it pathological within a few hours (31) and a few more treatments cause it to become markedly thickened, hyperemic, and hyperplastic, bringing it to a state highly favorable to the support of any tumors that may arise, as the active connective tissue participation in the developing growths sufficiently attests. Neoplastic changes occur relatively late and by the time they do so the conditions are ordinarily highly favorable to cell proliferation. Furthermore the first tumors to arise, the benign papillomas, have a native energy which renders them far less dependent on aid than those of the rabbit; they often undergo cancerous changes very soon; and many of them contain malignant cells almost from the start (32). The course of events in the rabbit much more nearly resembles that in human skin exposed to tar or other chemical carcinogens (33).

formation of growths. They are in other words *procarcinogenic*. It would seem well to reserve the term *cocarcinogenic* for agents which possess the ability to bring about neoplastic changes, and to work in concert with other carcinogens.

Cell Differences as Determining Species Differences to the Carcinogens.—The rabbit is supposed to be relatively refractory to carcinogenic agents (12), and certainly benzpyrene produces visible tumors on its skin much more slowly than on that of the mouse, for reasons already gone into. But with tar, which has a pronounced forcing influence on tumor cells, or even methylcholanthrene which has much less power of the sort, the rabbit proves the more responsive animal. In our experience tarring has sometimes caused papillomas and carcinomatoids to appear and reach a diameter of a centimeter within 10 days, after only three applications all told,¹² whereas the first growths in the mouse are not seen until after nearly 2 months, even when the entire back is tarred. Using methylcholanthrene we have obtained sizeable tumors on rabbit ears within 15 to 17 days, as already stated. In this general connection of species response it may be recalled that benzene as such sometimes evokes tumors in the rabbit, whereas in the mouse none has resulted from long exposure. All in all it would appear that the conversion of normal epidermal cells to tumor cells is more readily accomplished in the rabbit. The reverse is true of fibroblasts, sarcomas of the subcutaneous tissue appearing soon and almost regularly in mice injected with benzpyrene or methylcholanthrene, growths not obtained in the rabbit until one or more years have elapsed, and then rarely. It is plain that in weighing the responsiveness of various animal species to the carcinogens one must think strictly in terms of the type of cell exposed to them, not in that of the tissues as such, much less of the organism as a whole.

Scope of the Promoting Influence.—A discrimination of the initiating and promoting elements in tumor causation is essential not only to realization of the factors concerned in carcinogenic action, and for the appraisal of individual carcinogens, but because influences encouraging cell multiplication can on occasion be critical to the development of cancers.

As stated above, the healing of wounds in mouse skin previously exposed to a carcinogen may call forth cancers (28, 29). A serious hazard in the removal of x-ray cancers in man is the risk that a second cancer will appear where the pathological skin is healing. Almost all of the tar carcinomas of rabbits spring from the benign papillomas that this agent induces, as the earliest investigators noted, and since the papillomas are wholly dependent upon promoting influences for survival it follows that the occurrence of the derivative cancers is determined by these influences as well. The same often holds true of the tar cancers of man, these being usually preceded by papillomas, often in considerable number, which ordinarily retrogress,—especially if tarring is not kept up too long,—but from one or another of which a carcinoma may take origin (33). Tannenbaum's data (34) on the influence of a restricted diet in mice to

¹² In these cases actual oncogenesis must have been exceedingly swift, not the drawn out process it is generally held to be.

hinder or prevent the occurrence of spontaneous mammary tumors, and the cutaneous carcinomas and subcutaneous sarcomas due to benzpyrene, indicate that the proliferation of the tumor cells was held up, not the initiation of neoplastic change. In many human "precancerous states" the excited condition of the connective tissue at the time neoplastic change takes place should provide just the conditions to enable malignant cells to realize upon their possibilities. But needless to say the rôle of promoting factors diminishes in proportion as tumor cells are themselves intrinsically capable.

The determining effects of tar, benzpyrene, and methylcholanthrene upon the character and structure of the growths they produce are dealt with in an accompanying paper.

SUMMARY AND CONCLUSIONS

Benzpyrene brings about neoplastic changes in rabbit epidermis much sooner than has been supposed. The long interval that elapses before visible growths appear is due in the main to the relatively slight power of the carcinogen to encourage multiplication of the cells it renders neoplastic. Yet some slight power of this sort it has. Methylcholanthrene has somewhat more but not nearly so much as tar. It may initiate neoplastic changes within less than 17 days, as compared with less than 10 days for tar, but tumors due to it do not ordinarily appear until months after those called forth by tarring. All three agents give rise to growths of essentially the same kinds, but most of those due to benzpyrene and methylcholanthrene remain for a long while small, dry, and indolent whereas many of the tar tumors are fleshy, vigorous, and rapidly enlarging,—differences wholly consequent on differences in the ability to promote growth. Such ability is an important element in the effectiveness of carcinogens.

Tar and the polycyclic hydrocarbons cause many more cells to become tumor cells than give rise to visible growths. Benzpyrene is as effective in initiating neoplastic changes when dissolved in mineral oil as when in benzene, yet no tumors result from it until months after the benzene solution has given rise to them, the reason being that when in oil it is almost devoid of influence to encourage cell proliferation. Benzene itself has a very slight influence of the sort. Solvents may determine not only whether carcinogens initiate neoplastic change but may condition to a crucial degree the influence of these agents to encourage tumor formation.

Rabbit epidermis is much more responsive to carcinogenic influences than that of the mouse, as measured in terms of time taken to elicit benign neoplasms. Even benzene will call forth these growths from rabbit skin. In appraising the relative responsiveness to carcinogens of various animal species it is essential to reckon in terms of cells of identical type.

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EXPLANATION OF PLATES

PLATE 7

All of the sections were stained with methylene blue and eosin.

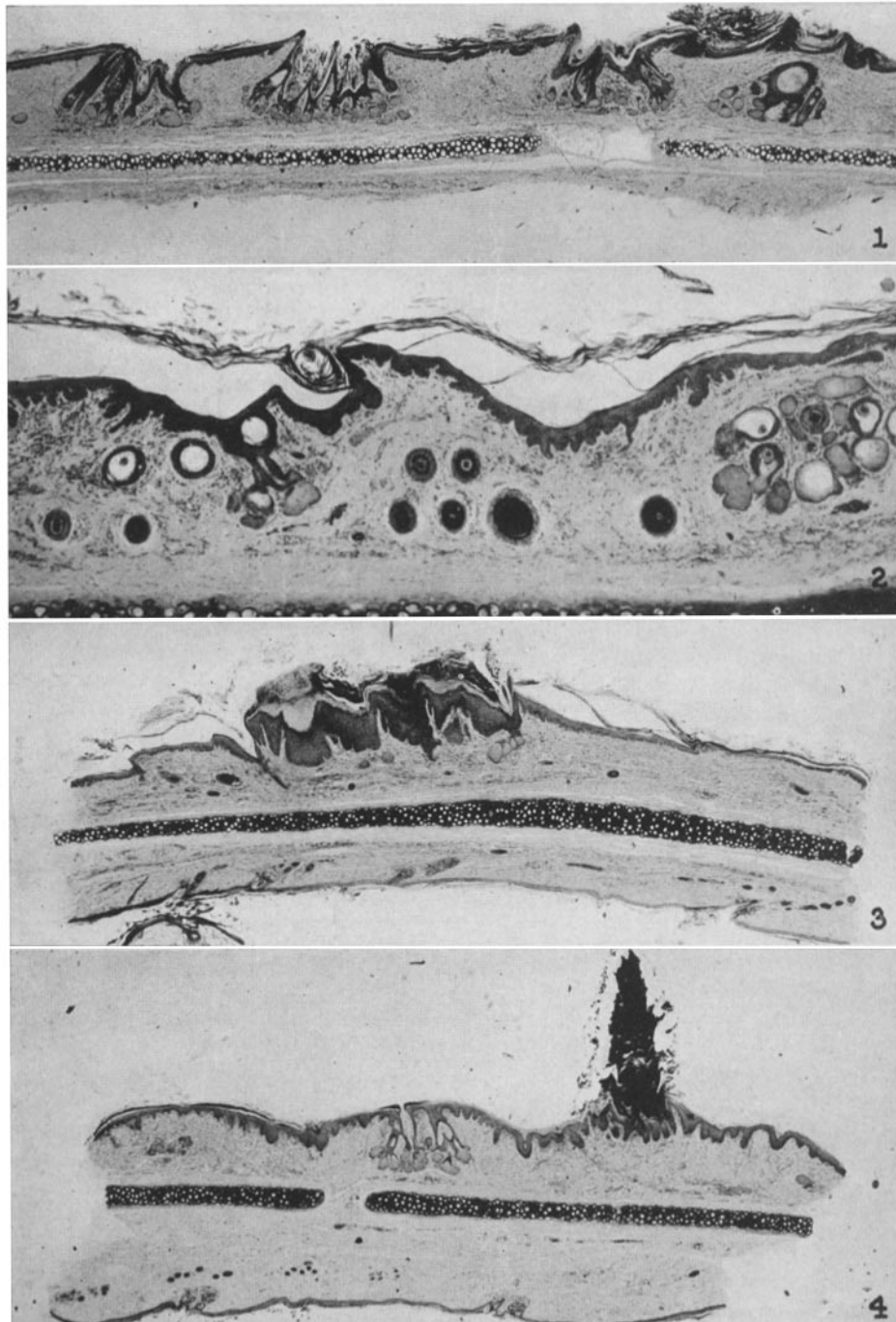
The photographs were made by Mr. Joseph B. Haulenbeek.

FIG. 1. Skin of the inner side of an ear after treatment with 0.3 per cent of benzpyrene over a period of 10 months. The epidermis is much thickened and hyperkeratotic, the hair follicles widened and cystic with accumulated keratin, the corium thickened and more cellular than usual, and the sebaceous glands are much increased in number. $\times 20$.

FIG. 2. State of the skin $4\frac{1}{2}$ months after benzpyrene applications were discontinued: they had been kept up for more than 15 months. The changes mentioned in Fig. 1 have persisted and at many spots the epidermis extends deep in an irregular fringe. $\times 55$.

FIG. 3. Plaque-shaped papilloma (11) on skin treated with benzene. It was discovered on the 320th day and when punched out had been present slightly more than 3 weeks. $\times 20$.

FIG. 4. Typical frill horn resulting from the application of benzene. It was first noted on the 264th day of treatment and was punched out 2 months later. The changes elsewhere on the skin are of the same sort as those caused by benzpyrene in benzene. $\times 16.5$.



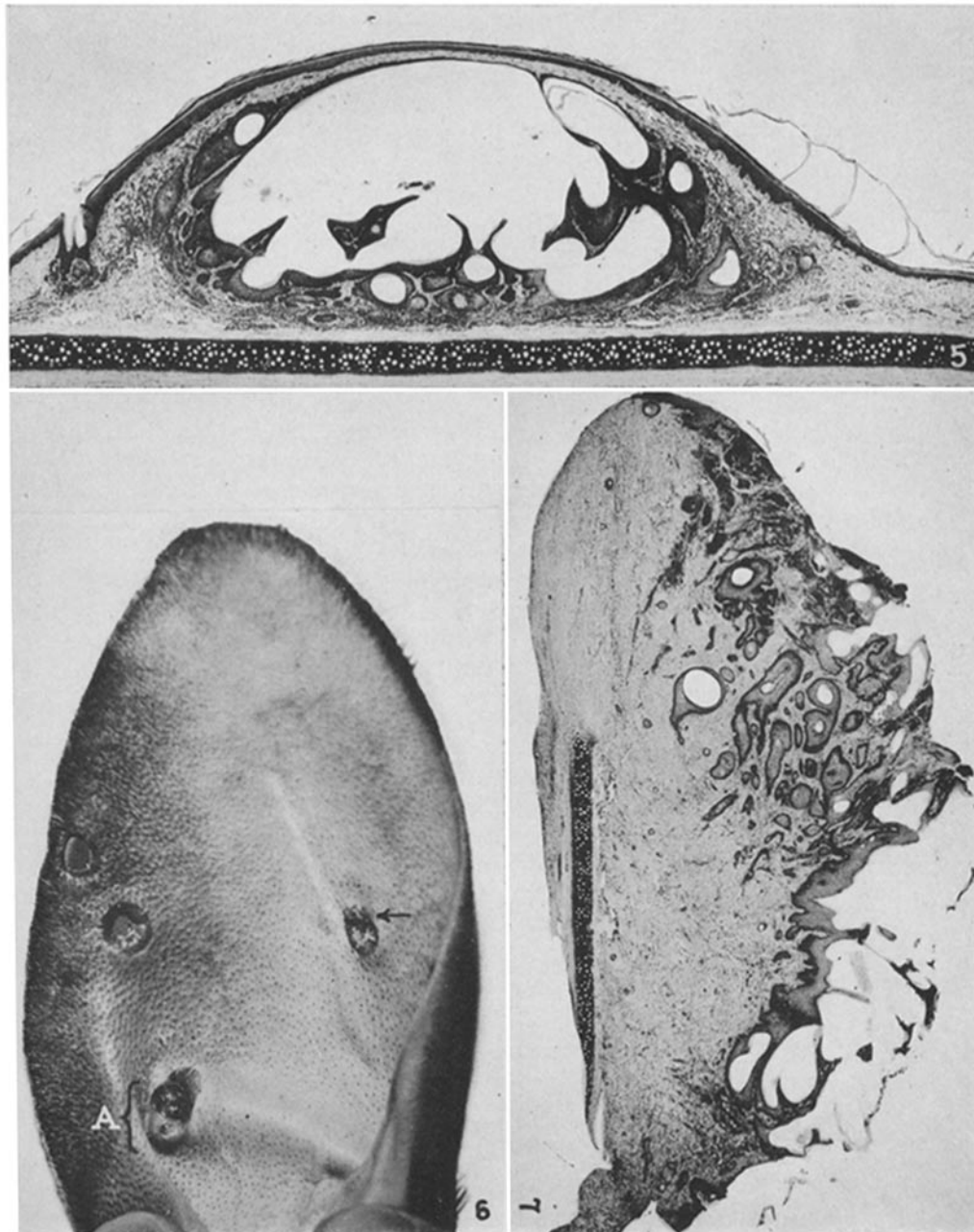
(Friedewald and Rous: Initiating and promoting elements in tumor production)

PLATE 8

FIG. 5. Unerupted carcinomatoid (or carcinoma?) from an ear receiving 0.3 per cent benzpyrene in mineral oil over a period of 10 months. There is a profuse round cell reaction about the growth, such as frequently accompanies retrogression. Presumably the tumor arose from hair follicle epithelium. $\times 20$.

FIG. 6. Tumors which arose where punch holes were healing in skin previously treated for 411 days with benzpyrene in mineral oil (D.R. 3-51, Chart 1). When the holes were made the ear carried 4 tiny discrete growths which were all removed by the punching, with a wide zone of tissue about them. None arose later except where the discing took place. Here 4 tumors appeared on the new tissue,—though only two (arrow and bracket) can be seen clearly in the photograph. This was taken on the 16th day of healing when none of the holes had completely closed. The part of the large growth *A* which lies on the new-formed disc has ulcerated and is dark with hemorrhage. The treated skin appears normal save for a slight widening of the follicle mouths. $\times 1$.

FIG. 7. Sagittal slice through the growth *A*, procured on the day after the photograph of Fig. 6 was taken. Toward the left it is mostly papillomatous though with anaplastic downgrowth near its edge, but on the right it has the aspect of a squamous cell carcinoma in which hemorrhages have occurred. $\times 13$.



(Friedewald and Rous: Initiating and promoting elements in tumor production)

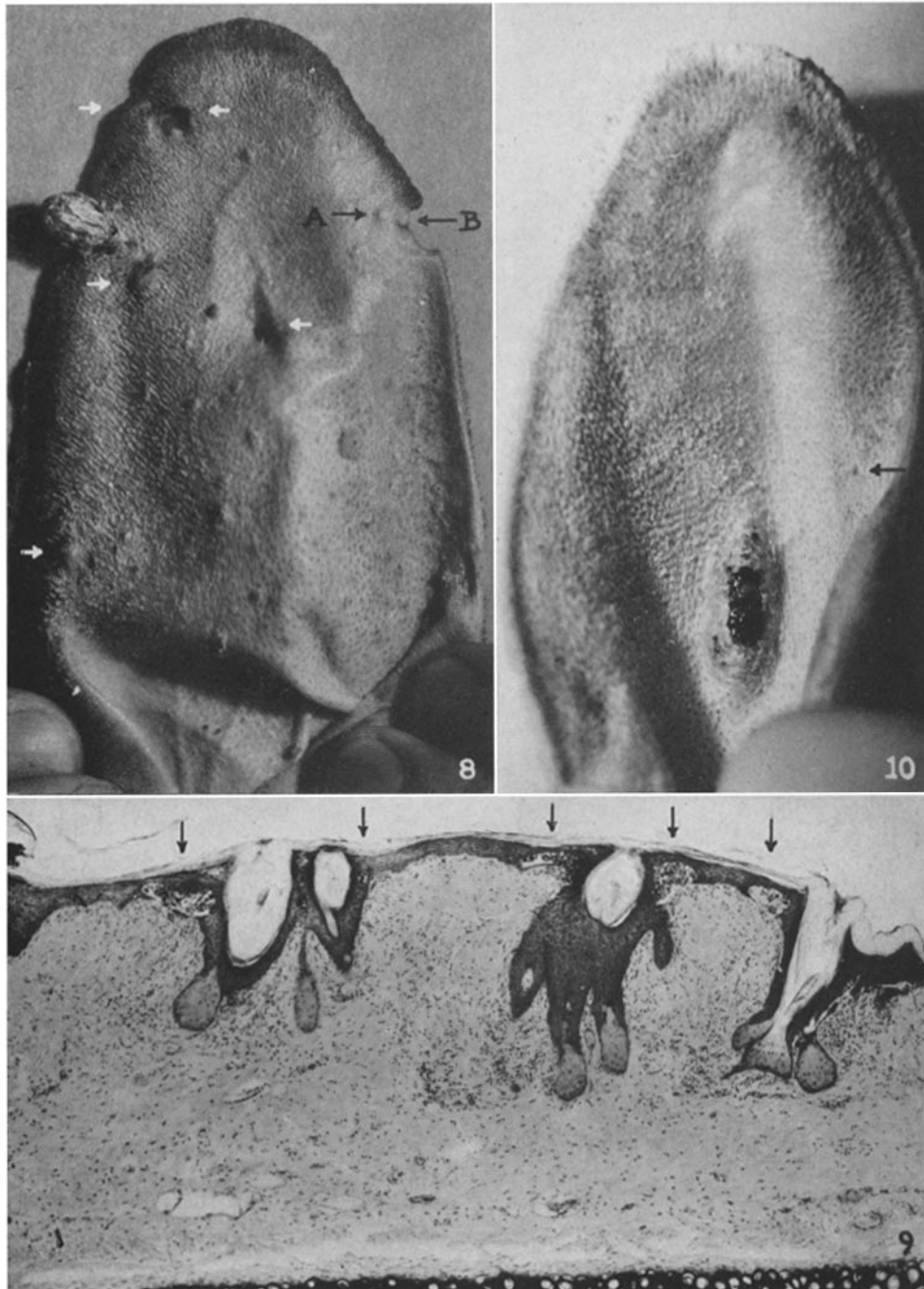
PLATE 9

FIG. 8. Tumors due to 0.3 per cent methylcholanthrene in benzene applied over a period of not quite 7 months. Similar findings were obtained with benzpyrene except that the growths arose much later and for a long time were not so numerous.

The skin is less scurfed than usual but shows a general distension of the follicle mouths with keratin. Most of the tumors are very small,—discrete low mounds and keratinized scabs,—but there is a single large, dry, vertically striated growth, a keratinizing papilloma. The white arrows point to spots where holes were once made and where there are now cuppings or bulgings due to excess proliferation of the cartilage. At the right margin of the ear healing of a semicircular biopsy wound is still incomplete and two growths can be seen (black arrows) which appeared soon after it began, one (*A*) at the edge of the original wound,—which is dimly visible as a crescentic line,—the other (*B*) at the edge of the new-formed tissue. $\times 1$.

FIG. 9. Blistering due to the application of a 0.3 per cent solution of methylcholanthrene in benzene over a period of 62 days. The blisters (arrows) lie next the distended openings of the hair follicles and involve mainly the Malpighian layer of the thickened epidermis, the epithelium beneath and over them still surviving in some places. The corium is abnormally cellular. $\times 55$.

FIG. 10. A carcinoma which appeared on an ear painted with methylcholanthrene in benzene for 12 weeks. The photograph was taken 2 weeks later and a punch biopsy on the next day showed the tumor to have the morphology of a moderately anaplastic squamous cell carcinoma. Elsewhere on the moderately scurfed ear there were several tiny growths at this time, but only one of them was big enough to be seen in the picture (arrow). During the weeks immediately after the biopsy the cancer almost entirely disappeared and then enlarged again, eventually involving most of the ear and metastasizing to a regional lymph node (see text). $\times 1$.



(Friedewald and Rous: Initiating and promoting elements in tumor production)